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SecDFyajC forms a heterotetrameric complex with YidC

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Summary

The *Escherichia coli* preprotein translocase is composed of a 'preprotein conducting channel' domain that consists of the peripherally bound translocation ATPase SecA and the heterotrimeric SecYEG membrane protein complex. SecD, SecF, and YajC form another heterotrimeric complex that can associate with the SecYEG complex. YidC is an essential membrane protein that plays a role in the integration of newly synthesized membrane proteins, and has been shown to co-purify with SecYEG when all translocase components are overproduced. Here, we demonstrate that under conditions that YidC co-purifies with overproduced SecDFyajC it does not co-purify with overproduced SecYEG. Moreover, this interaction of YidC with the SecDFyajC complex is also found at chromosomal protein levels of SecD, SecF and YajC. Closer examination of the SecDFyajC–YidC complex showed that YidC binds to SecD and SecF, whereas YajC interacts only with SecF. As SecF and YajC have previously been shown to interact with SecY, we propose that these two proteins link the heterotetrameric SecDFyajC–YidC complex to the SecYEG complex.

Introduction

The last decade has seen a major advance in our knowledge of the mechanistic role of the core components of the bacterial 'translocase', the membrane complex that transports the majority of secretory proteins across and inserts most membrane proteins into the bacterial cytoplasmic membrane (for a recent review, see Driessen *et al.*, 2001). The *Escherichia coli* translocase is composed of a peripheral ATPase, SecA and a heterotrimeric integral membrane domain with SecY, SecE and SecG as subunits. SecD, SecF and YajC form another heterotrimeric complex that can associate with the SecYEG

complex (Duong and Wickner, 1997a). YidC is an additional protein that associates with the translocase and which plays a role in the assembly of inner membrane proteins; (Samuelson *et al.*, 2000; Scotti *et al.*, 2000). Besides a role in assembly of 'Sec-dependent' membrane proteins, YidC also plays a role in the insertion of 'Sec-independent' membrane proteins (Samuelson *et al.*, 2000).

SecA serves both as a receptor for precursor proteins (preproteins) and as an ATP-driven molecular motor that directs the movement of a translocating preprotein. Upon binding of SecA and a preprotein, the trimeric SecYEG complex assembles into a tetrameric aqueous channel (Manting *et al.*, 2000) that accommodates a translocating preprotein together with at least part of the SecA molecule (Economou and Wickner, 1994). SecA undergoes large conformational changes upon binding and hydrolysis of ATP, and these allow the stepwise movement of a translocating polypeptide chain across the membrane (Schiebel *et al.*, 1991; van der Wolk *et al.*, 1997). The proton motive force stimulates protein movement by providing additional directionality to the process and can completely drive translocation at late stages (Schiebel *et al.*, 1991; Driessen, 1992).

Despite the progress made in recent years towards the understanding of the translocation mechanism and structural features of the translocase components, the role of the SecDFyajC complex in the translocation process remains unclear. A paradoxical aspect is that protein translocation can be reconstituted *in vitro* with purified SecA and SecYEG proteoliposomes (Brundage *et al.*, 1990; Hanada *et al.*, 1994), whereas cells lacking SecD and/or SecF are severely defective in protein export and barely viable (J. Pogliano and Beckwith, 1994). SecD and SecF have been identified via genetic screens for mutants that cause a pleiotropic export defect (Gardel *et al.*, 1987; Riggs *et al.*, 1988). Unlike other Sec-proteins, SecD and SecF are encoded by one operon that also contains the *yajC* gene (Gardel *et al.*, 1990). The latter encodes an 8 kDa protein that forms a complex with both SecYEG and SecDF (Duong and Wickner, 1997a), but which is not essential for cell viability and protein export (Pogliano and Beckwith, 1994). SecD and SecF are integral membrane proteins with six transmembrane segments and a large first periplasmic domain (about 45 and 11 kDa respectively) (Gardel *et al.*, 1990). In some bacteria, like *Bacillus subtilis* (Bolhuis *et al.*, 1998), SecD and SecF are

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Table 1. Complementation of *yajCsecDF* mutants.

plasmid	Strain Properties	Complementation		
		JP325 (P _{BAD} ⁺ - <i>yajCsecDF</i>)	KJ173 (<i>secD29cs</i>)	KJ184 (<i>secF62cs</i>)
pET606	<i>plac-yajCsecDF</i>	ND	+	+
pET529	<i>yajCsecDF</i>	+	+	+
pET543	<i>yajCsecDF</i> _{his6}	+	+	+
pET581	<i>secDF</i> _{his6}	+	+	+
pET547	<i>yajCsecF</i> _{his6}	—	—	+
pET554	<i>yajCsecD</i> _{his6}	—	+	—

ND, not determined.

Plasmids were transformed into the indicated strains. Complementation was tested by the ability of the plasmids to correct the growth defect of strain JP325 on glucose media or to correct the cold-sensitive growth defect of strains KJ173 (*secD29*) and KJ184 (*secF62*) (at 23°C). '+', plasmid complements the growth defect; '—', no complementation. *E. coli* strain JP325 is resistant to kanamycin. Therefore, complementation by plasmid pET606 could not be determined.

fused into a single polypeptide chain. In this arrangement, the structural architecture is similar to certain multidrug resistance RND (Resistance, Nodulation, Division)-type pumps with 12 transmembrane segments (i.e. AcrF and Act11-3) (Tseng *et al.*, 1999). Based on this structural homology, it is possible that the SecDF complex fulfils a transport function, such as the removal of cleaved signal sequences or transmembrane segments from the aqueous protein-conducting channel formed by the SecYEG complex. However, many other functions for the SecDF complex in protein translocation have been postulated. These are: (i) the SecDF complex is involved in the release of proteins from the translocase once they are translocated across the cytoplasmic membrane (Matsuyama *et al.*, 1993); (ii) the SecDF complex regulates SecA cycling (Economou *et al.*, 1995); and (iii) the SecDF complex prevents preprotein backsliding by slowing the movement of the translocating preprotein (Duong and Wickner, 1997b).

Here we show that the *E. coli* SecDFyajC complex interacts with YidC. The implications of this finding in relation to a role of SecDFyajC in protein transport are discussed.

Results

SecD and SecF are in low abundance in *E. coli* (less than 30 copies per cell; Matsuyama *et al.*, 1992; K. Pogliano and Beckwith, 1994). Hence, as a first step towards the characterization of the SecDFyajC complex, the *secDF* operon (containing the *yajC*, *secD* and *secF* genes) was cloned in different expression-controlled systems. First, to facilitate the cloning, unique restriction sites were introduced at the 5'- and 3'-ends of the *secDF* operon. Second, to simplify the purification of the SecDFyajC complex and to facilitate the analysis of the interaction between different subunits, C-terminal hexahistidine-tagged versions of SecD and SecF were constructed.

Both histidine-tagged SecD and SecF restored the growth of strains with a conditional lethal mutation in the *secD* or *secF* gene respectively (Table 1). Moreover, in the presence of a wild-type *secD* gene, histidine-tagged SecF was able to restore the growth of strain JP325 (see below) under SecDFyajC-depleting conditions (Table 1).

Overproduction of YidC suppresses the SecDFyajC[−] growth defect

In *E. coli* strain JP325, the chromosomal *secDF* operon is under control of the *araB* promoter. As a consequence, strain JP325 synthesizes YajC, SecD and SecF from the chromosome and forms colonies on agar plates only when grown in the presence of arabinose (Fig. 1A). While testing the functionality of the different constructed SecD-FyajC plasmids, we observed that besides plasmids containing both *secD* and *secF*, also overproduction of YidC from a plasmid restores the growth of strain JP325 under SecDFyajC-depleting conditions (Fig. 1A). To rule out that overproduction of YidC leads to a less efficient depletion of SecD and SecF on glucose media, the colonies were collected from the Luria–Bertani (LB)-agarose plate and membranes were isolated from these cells. Immunoblot analysis showed that the amount of SecD (data not shown) and SecF was below a detectable level and that YidC is highly expressed (Fig. 1B, lane 2). In contrast to suppression of the SecDFyajC[−] growth defect by a plasmid encoding the *secDF* operon, suppression by a plasmid containing *yidC* depends on the addition of IPTG to the growth medium. This indicates that overproduction of YidC is needed to overcome the growth defect of SecDFyajC depleted cells. Interestingly, whereas overproduction of YidC also suppressed the cold-sensitive phenotype of strains KJ173 (*secD29Cs*) and KJ184 (*secF62Cs*), overproduction of SecDFyajC does not restore the growth of cells depleted for YidC (data not shown).

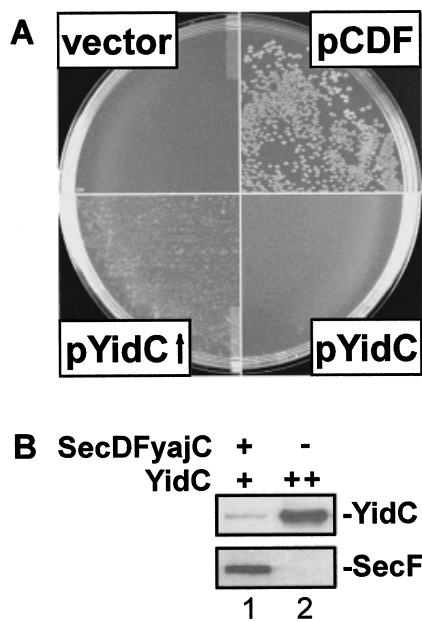


Fig. 1. Overexpression of YidC suppresses the SecDFyajC⁻ growth defect.

A. *E. coli* JP325 (P_{BAD}::yajCsecDF) transformed with either pCDF (pET578), pYidC (pET589) or an empty vector (pTrc99A) were grown till OD₆₀₀ = 0.8 on LB 0.2% arabinose media. Cells were harvested, washed with physiological salt solution and 150 µl of a 10⁻⁷ dilution was plated onto LB/ampicillin (100 µg ml⁻¹) containing 0.5% glucose or 0.5% glucose/0.5 mM IPTG (pYidC₋) and incubated at 30°C for 1 day.

B. Crude membranes of JP325/pYidC cells grown on LB plates containing 0.5% arabinose (lane 1) or 0.5% glucose/0.5 mM IPTG (lane 2) immunostained with antibodies against YidC and SecF.

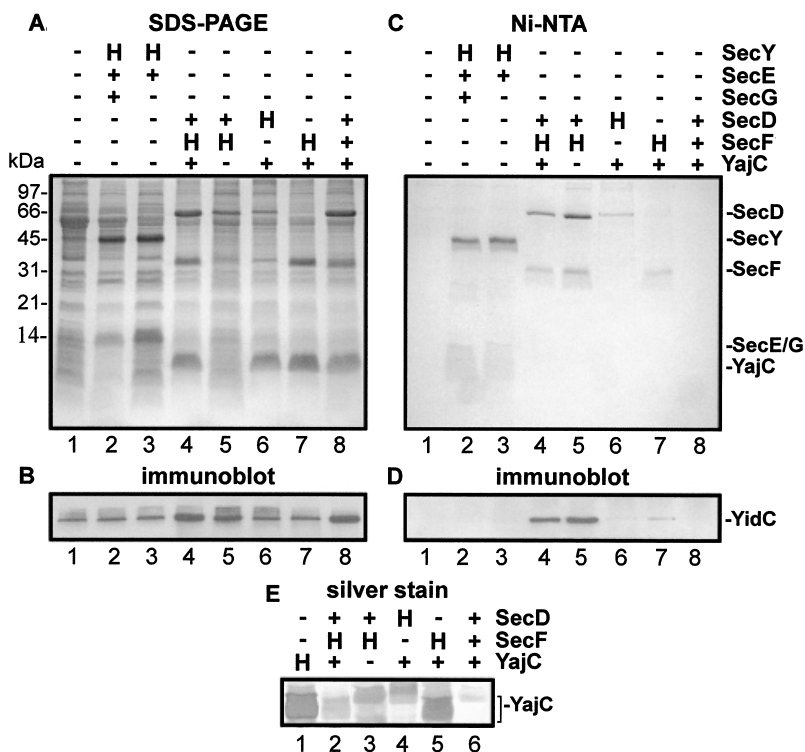


Fig. 2. YidC co-precipitates with the SecDFyajC complex. Inner membrane vesicles (IMVs) were prepared from *E. coli* SF100 cells overproducing different combinations of Sec proteins and analysed by SDS-PAGE and CBB staining (A) or electroblotted to PVDF membrane and immunostained with an antibody against YidC (B). After solubilization of the IMVs with dodecylmaltoside (DDM), the different Sec subcomplexes were purified with Ni²⁺-NTA beads and analysed by SDS-PAGE and CBB-staining (C) or by electroblotting and immunostaining using an antibody against YidC (D). The presence of YajC in the Ni²⁺-NTA-purified subcomplexes was analysed using SDS-PAGE and silver staining (E). '+', Sec protein expressed from plasmid; 'H', Sec protein expressed from a plasmid with a N- or C-terminal histidine tag.

YidC physically interacts with SecDFyajC and/or the other subcomplexes, IMVs were solubilized with the detergent dodecylmaltoside (DDM) and the different subcomplexes were purified by Ni^{2+} -NTA agarose chromatography (note that only one protein in an overproduced subcomplex contains a hexahistidine tag). Immunoblot analysis showed that YidC co-purifies with histidine-tagged SecF when SecDFyajC or SecDF are overproduced (Fig. 2D, lanes 4 and 5). Under these conditions, besides YidC, SecD and YajC also co-purify with histidine-tagged SecF (Fig. 2B, lanes 4 and 5; Fig. 2E, lane 2), indicating that SecD, SecF, YajC and YidC form a stable complex. Minor amounts of YidC co-purify with YajC-SecF_{his6} and SecD_{his6}, indicating that YidC weakly interacts with the individual SecD and SecF subunits (Fig. 2D, lanes 6 and 7). Interestingly, YidC does not co-purify with the overproduced SecYE(G) complex under these conditions (Fig. 2D, lanes 2 and 3).

To examine the interaction of YajC with SecD and SecF, the amount of YajC in the different purified subcomplexes was determined using silver staining. YajC only co-purified with complexes that contain SecF (Fig. 2E, lanes 2 and 5). However, it should be emphasized that the amount of YajC that co-purified with SecF or SecDF varied between experiments, indicating that the interaction of YajC with SecF and SecDF is loose.

A major fraction of the chromosomal encoded YidC associates with the overproduced SecDFyajC complex

To determine the fraction of YidC that co-purifies with the SecDFyajC complex, the complex was isolated by Ni^{2+} -NTA chromatography and the amount YidC in the flow-through and eluted fraction was analysed by immunoblotting. Whereas incubation with Ni^{2+} -NTA agarose beads almost quantitatively removes histidine-tagged SecF from the membrane extract, only 40–50% of the total amount of YidC is removed (Fig. 3A, lane 3). Immunoblot analysis further showed that under these conditions approximately 70% of the total amount SecD is removed from the membrane extract, and that the Ni^{2+} -NTA bound SecD and YidC co-elute from the beads with SecF (Fig. 3A, lane 4). Co-purification of SecD and YidC with hexahistidine-tagged SecF is very specific as neither SecD nor YidC was found to bind to the Ni^{2+} -NTA agarose beads when IMVs were used, which contained overproduced wild-type SecDFyajC (Fig. 3B). These data demonstrate that a major fraction of YidC interacts strongly with overproduced SecDF(yajC) and resists dissociation due to the detergent treatment.

SecDFyajC interacts with YidC at chromosomal protein levels

To assess whether the interaction of YidC with the

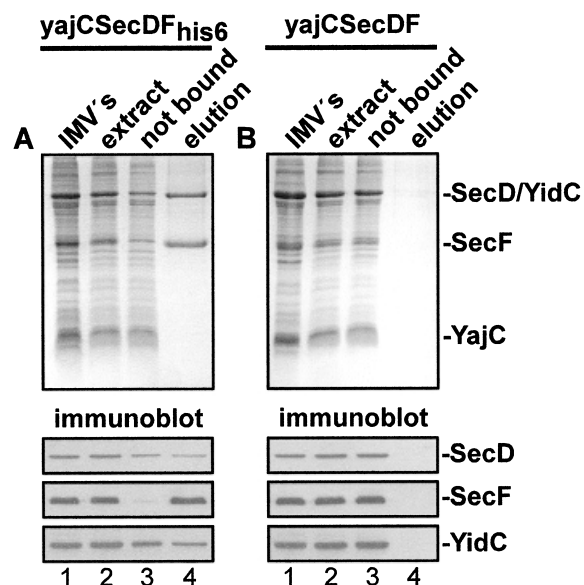


Fig. 3. 40–50% of chromosomal encoded YidC co-purifies with overproduced SecDFyajC. IMVs from *E. coli* SF100 cell containing overproduced histidine-tagged SecDFyajC (A) or wild-type SecDFyajC (B) were solubilized with DDM and incubated with Ni^{2+} -NTA beads. The fraction of SecD, SecF and YidC that binds to the Ni^{2+} -NTA agarose beads was determined using SDS-PAGE and CBB-staining, and by immunostaining using antibodies against SecD, SecF and YidC.

SecDFyajC complex was an artefact of the overproduction of these proteins, experiments were conducted using near to wild-type levels of SecDFyajC. For this purpose, a plasmid containing wild-type or hexahistidine-tagged SecDFyajC was transformed to strain JP325 (*tgt::kan-araC-P_{BAD}-yajCsecDF*). On glucose media, both plasmids complement the SecDFyajC[−] growth defect (Table 1) and the levels of SecD (data not shown), SecF and YidC in these cells are slightly elevated compared with wild-type cells (Fig. 4A). Using Ni^{2+} -NTA agarose beads, both SecD and YidC co-purify with histidine-tagged SecF (Fig. 4B, lane 2). This co-purification is very specific, as no SecD and YidC are detected when the same procedure is carried out with non-histidine-tagged SecDFyajC (Fig. 4B, lane 1). Therefore, we concluded that SecDFyajC and YidC also interact at near to chromosomal protein levels.

SecDFyajC forms a tetrameric complex with YidC

Wild-type SecDFyajC complex was purified from an overproducing strain via conventional cation exchange chromatography. Immunoblot analysis showed that the purified SecDFyajC complex also contained YidC (Fig. 5C, lane 1). As SecD and YidC have exactly the same migration behaviour on many different gel systems (unpublished results), it was impossible to determine the

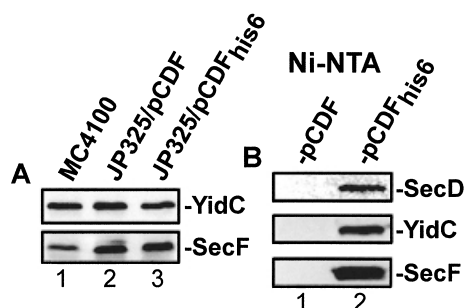


Fig. 4. YidC interacts with SecDFyajC at roughly chromosomal levels.

A. IMVs were prepared from wild-type *E. coli* strain MC4100, JP325/pET529 and JP325/pET543 grown on glucose media, and analysed using SDS-PAGE, blotted on PVDF membrane and immunostained with antibodies against SecF and YidC.
B. IMVs prepared from *E. coli* strain JP325/pET529 (lanes 1) and JP325/pET543 (lanes 2) grown on glucose media were solubilized with DDM and incubated with Ni²⁺-NTA beads as described in *Experimental procedures*. Eluted material was analysed by SDS-PAGE, blotted on PVDF membrane and immunostained with antibodies against SecD, SecF and YidC.

amount of YidC in the SecDFyajC preparation via CBB staining of the gel. However, on Blue Native PAGE, the purified complex migrated as a single band with a molecular mass of about 160 kDa (Fig. 5A, lane 1), whereas pretreatment of the sample with 1% SDS resulted in the dissociation of complex yielding protein bands of about 66, 40 and 8 kDa (Fig. 5A, lane 2). To examine the subunit composition of the 160 kDa protein band, the band was cut out and the gel slice was incubated with 1% SDS sample buffer. SDS-PAGE and silver staining of the

eluted material showed protein bands of about 66, 40 and 8 kDa (Fig. 5B, lane 2). The 66 kDa band reacted with both SecD and YidC antibodies, whereas the 40 kDa band reacted with an antibody against SecF (Fig. 5C). Moreover, the ~8 kDa band migrates at the same level as purified YajC (data not shown). Previously we have shown that purified YidC migrates as monomeric and dimeric species on BN-PAGE (van der Laan *et al.*, 2001; Fig. 5D, lane 2). Dimeric YidC migrates at a clearly distinct position from the ~160 kDa band (Fig. 5D), indicating that the presence of YidC in this large complex is not due to an accidental comigration of dimeric YidC with a complex of SecD, SecF and YajC. Taken together, these data demonstrate that SecD, SecF and YajC form a heterotetrameric complex with YidC.

As YidC and SecD comigrate on different gel systems, complex formation between YidC and SecDF(yajC) could be demonstrated only by immunoblotting. To exclude that the antibodies used cross-react, we purified YidC and a SecDF fusion protein. The SecDF fusion protein complemented the growth of the SecDF depletion strain (data not shown) but, as a small portion of the fusion protein was cleaved, we cannot ascertain that the complementation is due to the fusion protein. Immunoblot experiments using these purified proteins show that the YidC antibody does not react with the SecDF fusion protein (Fig. 6B, lane 3) and that the SecD antibody does not react with the YidC protein (Fig. 6C, lane 1). In contrast, both antibodies react with the ~66 kDa band present in the purified SecDFyajC preparation (Fig 6B and C, lane 2). This clearly demonstrates that the antibodies used in this study are specific and that YidC forms a complex with SecDFyajC.

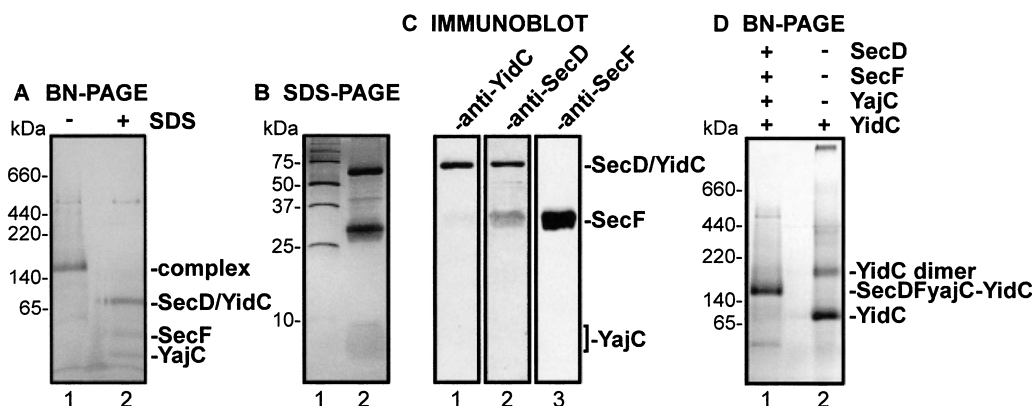


Fig. 5. Blue Native PAGE of purified SecDFyajC.

A. Purified SecDFyajC (20 µg) was incubated for 15 min at room temperature in the presence or absence of 1% SDS and loaded on a 5–15% BN-PAGE. After electrophoresis, the gel was stained with Coomassie brilliant blue R250.
B. The ~160 kDa protein band seen on BN-PAGE was cut out and the gel slice was incubated with 1% SDS sample buffer. Proteins eluted from the gel slice were analysed by SDS-PAGE and silver staining or after SDS-PAGE transferred to a PVDF membrane and immunostained with antibodies against SecD, SecF and YidC (C). Note: as the SecF blot was stripped from antibody and re-probed with the SecD antibody, a weak reaction with SecF remained.
D. BN-PAGE of purified SecDFyajC–YidC complex and purified YidC.

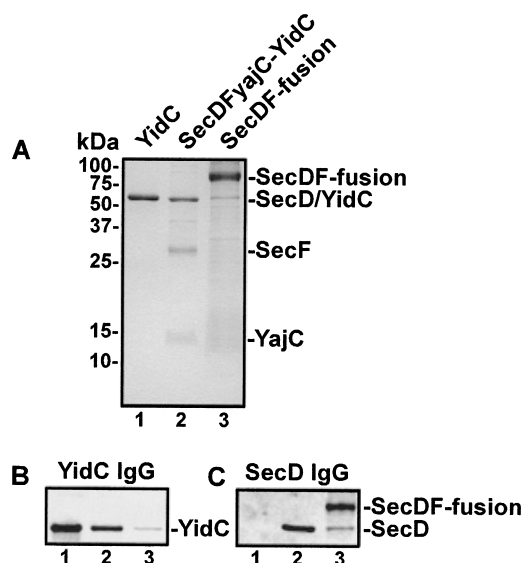


Fig. 6. SecD and YidC antibodies are specific.

A. Purified YidC, SecDFyajC–YidC complex and a SecD fusion protein were loaded on 12% SDS–PAGE and stained with CBB or blotted on PVDF membrane and immunostained with antibodies against YidC (B) and SecD (C).

Discussion

Previously, we have shown that a small fraction of YidC co-purifies with SecYEG when all translocase components (i.e. SecYEG and SecDFyajC) are overexpressed (Scotti *et al.*, 2000). In this report, we show that under conditions that YidC interacts with overproduced SecDF(yajC) it does not interact with the overproduced SecYE(G) complex. Moreover, this interaction of YidC with the SecDFyajC complex is also observed at near to chromosomal levels of SecD, SecF and YajC. Genetic and biochemical data indicate that SecDFyajC forms a complex with SecYEG (Sagara *et al.*, 1994; Duong and Wickner, 1997a). As YidC forms a stable complex with SecDF, we propose that the latter functions as the scaffold that links YidC to the SecYEGDFyajC complex. Consequently, depletion of SecDFyajC directly affects the function of YidC in SecYEG-dependent membrane protein insertion. As YidC is an essential protein (Samuelson *et al.*, 2000), an impaired interaction between YidC and SecYEG might be the main reason for the growth defect observed in SecDFyajC[−] cells. Along these lines, it is easy to envisage why overproduction of YidC is able to restore a SecDFyajC[−] growth defect as it increases the chance that a YidC molecule is in the vicinity of a SecYEG complex by mass action.

Our data do not provide a mechanistic role for SecDFyajC in protein translocation or membrane protein insertion. However, our new finding that SecDFyajC and YidC form a stable complex implicates that some previous speculations on the role of the SecDFyajC complex needs

to be re-evaluated. First, overproduction of SecDFyajC has been shown to suppress some signal sequence mutations (J. Pogliano and Beckwith, 1994). This suppressing effect has been attributed to the regulatory function of SecDFyajC on the SecA insertion cycle (Economou *et al.*, 1995). However, from an evolutionary point of view it is questionable whether SecDFyajC directly affects the SecA membrane cycling as euryarcheotes contain homologues of SecD and SecF (Pohlschroder *et al.*, 1997; Eichler, 2000), but lack a homologue of SecA. Moreover, in this paper we show that overproduction of SecDFyajC results in an increased YidC level in the cell. As YidC has been shown to interact with the hydrophobic transmembrane segment of membrane proteins (Houben *et al.*, 2000; Scotti *et al.*, 2000), it is reasonable that YidC also interacts with hydrophobic signal sequences. This interaction is expected to stabilize the membrane-inserted signal sequence. It is therefore plausible that not SecDFyajC itself but the elevated level of YidC in cells overproducing SecDFyajC restores translocation of preproteins with a defective signal sequence. This hypothesis is currently under investigation. Second, the SecDFyajC complex has been shown to prevent backsliding of proOmpA translocation intermediates during translocation (Duong and Wickner, 1997b). This stabilizing effect has also been attributed to the regulating effect of SecDFyajC on the SecA membrane cycling. It is noteworthy that in those experiments only translocation intermediates that correlate to short hydrophobic stretches in proOmpA (Sato *et al.*, 1997) are stabilized by SecDFyajC (Duong and Wickner, 1997b). As YidC interacts with hydrophobic transmembrane segments and forms a part of the SecDFyajC–YidC complex, we propose that the YidC protein recognizes the short hydrophobic stretches in proOmpA thereby pausing and/or stabilizing proOmpA at specific positions.

Besides a function in Sec-dependent membrane protein insertion, YidC is also required for the Sec-independent membrane insertion of M13 procoat (Samuelson *et al.*, 2000). In this respect, it should be noted that under conditions in which SecDFyajC is overproduced, 40–50% of the chromosomally encoded YidC co-precipitates with histidine-tagged SecF. Moreover, at chromosomal levels, YidC is much more abundant than SecD and SecF (Urbanus *et al.*, 2002). This suggests that in the cell two forms of YidC exist; one that is in complex with SecDFyajC and a second form that may be free or loosely associated with other components. Future studies should reveal if there is free exchange between both YidC populations or if they function in two separate membrane protein insertion pathways.

Our overproduction and co-precipitation data of different combinations of SecD, SecF and YajC confirm and extend data from other groups, and provide further insight

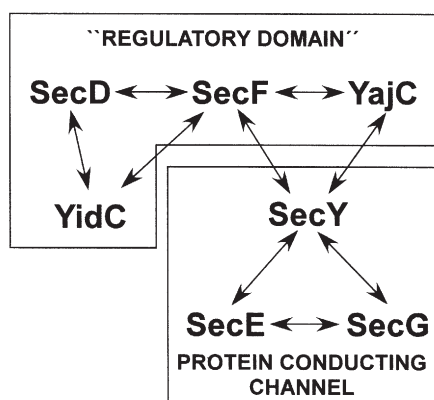


Fig. 7. Model for the interactions between the different subunits in the preprotein translocase.

in the association of the different subunits within the SecYEGDFyajC–YidC super-complex. Although small amounts of YidC co-purify with separate overproduced SecD and SecF, only co-overproduction of SecD and SecF resulted in an increased YidC level in the cell and an efficient co-purification with the SecDF complex. In addition, co-overproduction of SecF is needed for overproduction of SecD (Sagara *et al.*, 1994; Fig. 3A, lane 6). These results confirm that SecF associates with SecD and now demonstrate that this interaction is required for an efficient binding of the YidC protein. Although YajC is not essential for cell viability or protein translocation (Pogliano and Beckwith, 1994), it associates with the SecDF complex (Sagara *et al.*, 1994; Duong and Wickner, 1997a), and this association is specific for SecF (this paper). These results further link the YajC protein to the translocase, but the function of this small membrane protein remains to be elucidated. Previous studies also

indicated that both SecF and YajC interact with SecY (Sagara *et al.*, 1994; Duong and Wickner, 1997a), whereas SecY interacts with SecE (Taura *et al.*, 1993; Homma *et al.*, 1997), and SecG interacts with both SecY and SecE (Nishiyama *et al.*, 1995). We therefore propose that SecF and YajC connect the SecDFyajC–YidC complex with the SecYEG–SecA complex (summarized in Fig. 7).

Experimental procedures

Strains and plasmids

Escherichia coli strain JP325 (*ara*Δ714, Δ[*argF-lac*]U169, *rpsL*150, *relA*1, *thi*, *flb*5301, *deo*C1, *pts*F25, *recA*::cat, *tgt*::kan-*araC* ± P_{BAD}::*yajCsecDF*) (Economou *et al.*, 1995) was used for complementation studies, and strain SF100 (F[–], Δ*lacX*74, *galK*, *thi*, *rpsL*, *strA* Δ*phoA*(pvull), Δ*ompT*) (Baneyx and Georgiou, 1990) was used for overexpression of translocase components. The plasmids used in this study are listed in Table 2. To facilitate the characterization and expression of the *secDF* operon, we modified the chromosomal region containing *yajCsecDF* in the following way. First, we cloned the *SspI*–*SfuI* fragment of pCGSH1 into the *Bam*HI site of pUC18. Second, to ease subcloning into different expression vectors, a unique *NcoI* site was introduced at the initiation codon of *yajC* and a unique *Hind*III site just after the *secF* gene. Third, to facilitate purification of the overproduced proteins, C-terminal hexahistidine-tagged versions were made of SecD and SecF. Using these mutants, plasmids were constructed that lack one of the genes of the operon in combination with a histidine-tagged version of SecD or SecF. For overexpression of YidC, the *XbaI*–*Hind*III fragment of pET1819 (gift of Jelto Swaving) was cloned into expression vector pTrc99A (Amann and Brosius, 1985). All plasmids were sequenced and tested for the ability to complement *secD*, *secF* or *yidC* mutants.

Table 2. Plasmids.

Plasmid	Relevant characteristics	Source or reference
pTrc99A	Expression vector, <i>trc</i> promoter, Amp ^r	Amann and Brosius (1985)
pBAD18	Expression vector, <i>araB</i> promoter, Amp ^r	Guzman <i>et al.</i> (1995)
pET610	<i>SecYEG</i> behind <i>trc</i> promoter with N-terminal His-tagged SecY, Amp ^r	van der Does <i>et al.</i> (1998)
pET320	<i>SecYE</i> behind <i>trc</i> promoter with N-terminal His-tagged SecY, Amp ^r	van der Does <i>et al.</i> (2000)
pCGSH1	4.4 kb chromosomal region including <i>yajCsecDF</i> in pBR322	Gardel <i>et al.</i> (1990)
pET606	<i>yajCsecDF</i> behind <i>lac</i> -promoter, Kan ^r	Andreas Kaufmann
pET529	<i>SspI</i> – <i>SfuI</i> fragment pCGSH1 in pUC18, Amp ^r	This work
pET543	<i>yajCsecDF</i> with C-terminal His-tagged SecF in pUC18, Amp ^r	This work
pET581	<i>secDF</i> with C-terminal His-tagged SecF in pUC18, Amp ^r	This work
pET547	<i>yajCsecF</i> with C-terminal His-tagged SecF in pUC18, Amp ^r	This work
pET554	<i>yajCsecD</i> with C-terminal His-tagged SecD in pUC18, Amp ^r	This work
pET578	<i>yajCsecDF</i> behind <i>trc</i> promoter, Amp ^r	This work
pET575	<i>yajCsecDF</i> behind <i>araB</i> promoter, Amp ^r	This work
pET546	<i>yajCsecDF</i> behind <i>araB</i> promoter with C-terminal His-tagged SecF, Amp ^r	This work
pET584	<i>secDF</i> behind <i>araB</i> promoter with C-terminal His-tagged SecF, Amp ^r	This work
pET552	<i>yajCsecF</i> behind <i>araB</i> promoter with C-terminal His-tagged SecF, Amp ^r	This work
pET560	<i>yajCsecD</i> behind <i>araB</i> promoter with C-terminal His-tagged SecD, Amp ^r	This work
pET589	<i>yidC</i> behind <i>trc</i> promoter, Amp ^r	This work
pNN220	<i>yajCsecDF</i> -fusion behind <i>trc</i> promoter with C-terminal His-tagged SecDF, Amp ^r	This work

Co-precipitation studies

Inner membrane vesicles (IMVs) containing different combinations of overproduced Sec-proteins were solubilized in buffer A (25 mM Tris-HCl pH 8.0, 10% [v/v] glycerol, 100 mM NaCl, 2% [w/v] dodecylmaltoside [DDM]). After 1 h on ice, the insoluble material was removed by ultracentrifugation (30 min 100 000 g) and the supernatant was mixed with Ni²⁺-NTA agarose beads equilibrated with buffer B (25 mM Tris-HCl pH 8.0, 10% [v/v] glycerol, 100 mM NaCl, 0.1% [w/v] DDM, 10 mM imidazole). The suspension was gently shaken at 4°C and after overnight incubation the agarose beads were pelleted by centrifugation. The beads were washed twice with buffer B and bound proteins were eluted with buffer C (25 mM Tris-HCl pH 8.0, 10% [v/v] glycerol, 100 mM NaCl, 0.1% [w/v] DDM, 50 mM EDTA). Eluted material was analysed using SDS-PAGE and immunoblotting.

Overexpression and purification of SecDFyajC

An overnight culture of *E. coli* SF100 carrying pET606 was diluted 25-fold into fresh LB-medium supplemented with 50 µg ml⁻¹ of kanamycin and grown for 7 h at 37°C. Cells were disrupted by passage through a French press and IMVs were isolated as described (Kaufmann *et al.*, 1999). IMVs were solubilized in 50 mM Hepes/NaOH pH 7.0, 20% [v/v] glycerol, 2% [w/v] DDM at a protein concentration of 3 mg ml⁻¹. After 1 h, insoluble material was removed by ultracentrifugation (30 min at 100 000 g) and the clarified extract was loaded onto a Hi-Trap S-Sepharose cation exchange column (5 ml) equilibrated with buffer 50 mM Hepes/NaOH pH 7.0, 20% [v/v] glycerol, 0.1% [w/v] DDM. The column was rinsed with eight volumes of buffer A and eluted with a gradient of 0–0.5 M NaCl. Fractions containing pure SecDFyajC were pooled, frozen in liquid nitrogen and stored at –80°C.

Blue Native PAGE

Blue Native PAGE analysis of the purified SecDFyajC complex was performed on 5–15% gradient gels as described (Schagger *et al.*, 1994). To analyse the subunit composition of a protein band on BN-PAGE, proteins were eluted with 1% SDS sample buffer and analysed by SDS-PAGE and immunoblotting.

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